DNMT1 is predictive of survival and associated with Ki-67 expression in R-CHOP-treated diffuse large B-cell lymphomas

Suet Kee Loo[1], Ewe Seng Ch'ng[2], Charles H. Lawrie[3,4], María Arestin Muruzabal[4], Ayman Gaafar[5], María Puente Pomposo[6], Azlan Husin[7], Md. Salzihan Md. Salleh[8], Alison H. Banham[3], Lars M. Pedersen[9], Michael B. Møller[10], Tina M. Green[10], Kah Keng Wong[1]

[1]Department of Immunology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kelantan, Malaysia.
[2]Advanced Medical and Dental Institute, Universiti Sains Malaysia, Bertam, 13200 Kepala Batas, Pulau Pinang, Malaysia.
[3]Nuffield Division of Clinical Laboratory Sciences, Radcliffe Department of Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom.
[4]Oncology Department, Biodonostia Research Institute, San Sebastian, 20014, Spain.
[5]Department of Pathology, Hospital Universitario Cruces, Barakaldo, Spain.
[6]Department of Hematology, Hospital Universitario Cruces, Barakaldo, Spain.
[7]Department of Medicine, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kelantan, Malaysia.
[8]Department of Pathology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kelantan, Malaysia.
[9]Department of Haematology, Herlev University Hospital, Copenhagen, Denmark.
[10]Department of Pathology, Odense University Hospital, Odense, Denmark.

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Corresponding author
Dr. Kah Keng Wong (BSc Hons, Mal; DPhil, Oxon)
Department of Immunology, School of Medical Sciences,
Health Campus, Universiti Sains Malaysia,
16150 Kubang Kerian, Kelantan, Malaysia
E-mail: kahkeng@usm.my ; Tel: +609 7676229 ; Fax: +609 7653370

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ABSTRACT

DNMT1 is a target of approved anti-cancer drugs including decitabine. However, the prognostic value of DNMT1 protein expression in R-CHOP-treated diffuse large B-cell lymphomas (DLBCLs) remains unexplored. Here we showed that DNMT1 was expressed in majority of DLBCL cases (n=209/230; 90.9%) with higher expression in GC B-cell-like (GCB)-DLBCL subtype. Low and negative DNMT1 expression (20% cut-off; n=33/230; 14.3%) was predictive of worse overall survival (OS; P<0.001) and progression-free survival (PFS; P<0.001). Nonetheless, of the 209 DNMT1-positive patients, 33% and 42% did not achieve 5-year OS and PFS, respectively, indicating that DNMT1-positive patients showed considerably heterogeneous outcomes. Moreover, DNMT1 was frequently expressed in mitotic cells and significantly correlated with Ki-67 or BCL6 expression (r=0.60 or 0.44, respectively; P<0.001). We demonstrate that DNMT1 is predictive of DLBCL patients' survival, and suggest that DNMT1 could be a DLBCL therapeutic target due to its significant association with Ki-67.

Keywords: Diffuse large B-cell lymphoma, DNMT1, survival, Ki-67, BCL6

1.0 INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is an aggressive subtype of mature B-cell neoplasm[1] where approximately 30-40% of the patients develop relapsed/refractory disease despite the use of the standard immunochemotherapy regimen R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone).[2] Gene expression profiling (GEP) has identified at least two clinically and biologically distinct DLBCL subtypes: germinal center (GC) B-cell-like DLBCL (GCB-DLBCL) and activated B-cell-like DLBCL (ABC-DLBCL) subtypes,[3] with ABC-DLBCL patients exhibiting worse outcomes in response to the R-CHOP regimen.[4, 5]

Altered epigenetic regulation including methylation profiles is a recognized feature of DLBCL.[6] Molecular subtypes of DLBCL (ABC- or GCB-DLBCL) exhibit distinct DNA methylation profiles,[7] and greater magnitude of DNA methylation changes from normal GC B cells is associated with worse outcomes in R-CHOP-treated DLBCL patients.[8] DNA methylation of gene promoters at CpG sites and intragenic regions is mediated by DNA methyltransferases (DNMTs): DNMT1, DNMT3A, and DNMT3B. Of the DNMTs, DNMT3A and DNMT3B mediate *de novo* DNA methylation by establishing methylation of unmodified DNA, while DNMT1 maintains the methylation pattern by preferentially methylating the nascent unmethylated strand during cell replication and DNA synthesis.[9]

Studies using murine models have shown that Dnmt1 is required for GC formation and Dnmt1 hypomorphic mice show increased DNA damage in GC B cells.[10] Moreover, Dnmt1 is required to sustain T-cell lymphomas by preventing apoptosis in a murine MYC-induced T-cell lymphoma model.[11] In terms of DLBCL, DNMT1 protein was overexpressed in a series of DLBCL cases treated with CHOP-like regimens with concomitant expression of both DNMT1 and DNMT3B being correlated with treatment resistance.[12] Priming of high-risk DLBCL patients with azacytidine, that mainly targets DNMT1 and DNMT3, followed by R-CHOP treatment have been

shown to achieve a high complete remission rate,[13] and decitabine (targets DNMT1 and DNMT3) has recently completed phase I trials (ClinicalTrials.gov identifier: NCT00109824 and NCT00275080) investigating the efficacy and safety of the drug in DLBCL.

However, it is unknown whether DNMT1 expression confers prognostic value in DLBCL patients treated with R-CHOP. In this study, we set out to investigate the expression pattern of DNMT1 in DLBCL cases and its correlation with clinico-demographic parameters and survival in a multi-centre series of R-CHOP-treated DLBCL patients (n=230).

2.0 MATERIALS AND METHODS

2.1 Patient samples

The R-CHOP-treated primary DLBCL cases (n=230) have been described previously, with 40 cases from Spain, 33 cases from Malaysia,[14] and another 157 cases from Denmark.[15] Immunostaining was performed with other markers previously.[16-19] Clinico-demographic data of all patients are listed in Table 1. The cohort consisted of 133 males and 97 females (1.37:1 ratio) with median age of 64 years (20–91 years), and median OS and PFS (1–147 months for both) of 57 and 49 months, respectively. Reactive tonsil control tissues were obtained from the FFPE tissue archive of Department of Pathology, Universiti Sains Malaysia. All FFPE tissues used in this study were obtained in accordance with the Declaration of Helsinki and the study was approved by local ethics committees.

2.2 Immunohistochemistry (IHC)

IHC staining was performed using the EnVision kit (DakoCytomation, Glostrup, Denmark). Primary antibodies used, dilution factors and other IHC parameters are listed in Supplementary Table 1. DNMT1 protein expression [anti-DNMT1 antibody clone 2B5 (Abcam, Cambridge, UK)] was scored for frequency and intensity of positive tumor cells only. Frequency in 10% increment

was scored, and staining intensity was defined as follows: 1: negative; 2: weak/moderate; 3: strong. For intensity scoring, weak and moderate intensities could not be robustly distinguished and hence these two intensities were considered to be in the same group for a three-tier (*i.e.* negative, weak/moderate, or strong) intensity system as adopted by other studies.[20, 21] All cases were scored by a histopathologist (E.S.C.) and independently by two researchers (K.K.W. / S.K.L.). Cases with discrepant scores (*i.e.* differed by ≥20% frequency and/or different intensity) were resolved after joint examination (E.S.C. / S.K.L.) on a multi-headed microscope. The averaged and rounded to the nearest tenth frequency of both scorings and scoring of higher intensity were selected as the final scores. The reproducibility of interobservers' agreement on DNMT1 frequency was examined by Cronbach's α analysis (SPSS Statistics v22; Chicago, IL, USA) with α value of 0.942, indicating high reproducibility (α >0.9).[22]

The DLBCL cases were previously subtyped into GCB-DLBCL or non-GCB DLBCL according to Hans[23] or Visco-Young[24] algorithms for the Spanish and Malaysian series,[14] and Danish series[15] with the following monoclonal antibodies (mAbs): anti-CD10 mAb clone 56C6 (DakoCytomation), anti-BCL6 mAb clone PG-B6p (DakoCytomation) and clone LN22 (Leica Microsystems, Ballerup, Denmark) for the former and latter series, respectively, anti-MUM1 mAb clone MUM1p (DakoCytomation), and anti-FOXP1 mAb clone JC12 (Abcam).

2.3 Statistical analysis

Patients' clinico-demographic parameters according to DNMT1 expression were compared using the chi-square test for categorical variables, and the Mann–Whitney U-test for continuous variables. Correlation coefficients were assessed by Pearson (r) or Spearman (ρ) correlation test and $P<0.05$ was considered as significant (SPSS Statistics v22). Normality of DNMT1 expression was assessed using the histogram (GraphPad Prism v6.07; La Jolla, CA, USA) and coefficient of variation (CV; SPSS Statistics v22 software). The OS and PFS were analyzed

using the Kaplan–Meier method and comparison between survival curves were examined using the log-rank test (GraphPad Prism v6.07). Multivariate analysis was conducted using Cox's proportional hazards model (SPSS Statistics v22).

3.0 RESULTS

3.1 DNMT1 is Highly Expressed in Germinal Centers of Lymphoid Follicles

We set out to determine the optimal primary anti-DNMT1 antibody that produces specific staining by IHC, and four distinct antibody clones were tested on reactive tonsils. The polyclonal ab19905 and monoclonal clone 2B5 antibodies (both from Abcam, Cambridge, UK) yielded strong and specific staining of the GCs, while weak or absence of staining was observed in tonsils immunostained with the other two antibodies (clone 60B1220.1 from Abcam; clone 18/DNMT1 from BD Biosciences, San Jose, CA) even when tested at low dilutions (Supplementary Fig. 1). We adopted the mAb clone 2B5 (referenced as anti-DNMT1-2B5) for subsequent experiments as mAbs represent a constant and renewable resource for future studies. In secondary lymphoid follicles of reactive tonsil, DNMT1 was frequently and strongly expressed in the nuclei of B-cell centroblasts of the GC dark zone (DZ) with lower frequency and intensity in the GC light zone (LZ) (Fig. 1A) consistent with the immunostaining results for DNMT1 in tonsillar follicles by Shaknovich *et al*.[10]

3.2 DNMT1 is Frequently Expressed in DLBCL with Higher Expression in the GCB-DLBCL Subtype

We examined DNMT1 protein expression in a multi-center series of R-CHOP-treated primary DLBCL cases (n=230) with the anti-DNMT1-2B5 mAb, and found that DNMT1 was frequently expressed in DLBCL with variable frequency and intensity, and all cases showed nuclear expression (Fig. 1B). DNMT1 expression followed a non-Gaussian distribution according to visualization of the histogram that exhibited a skewed curve with the majority (n=128/230;

55.7%) of the cases displaying ≥60% frequency DNMT1 positivity, and coefficient of variation (CV) was calculated to be 56% (*i.e.*, above the 50% cut-off) denoting non-Gaussian distribution (Fig. 1C). In terms of staining intensity, 21 (9.1%), 136 (59.1%) and 73 (31.8%) cases displayed negative, weak/moderate and strong DNMT1 intensity, respectively (Fig. 1D).

In terms of association with clinico-demographical parameters, higher DNMT1 frequency (≥20% cut-off) was significantly associated with lower ECOG scores (P<0.05) but not associated with the rest of the parameters (Fig. 2A). DLBCL cases with strong DNMT1 intensity displayed significant relationship with lower ECOG scores (P<0.05) and the GCB-DLBCL subtype (P<0.001; Fig. 2B-C).

When compared in terms of continuous variables, higher DNMT1 frequency was associated with the GCB-DLBCL subtype (P=0.001) (Fig. 2D). However, DNMT1 was also frequently expressed in the non-GCB DLBCL subtype and the significant relationship with GCB-DLBCL subtype was mainly due to a higher proportion of GCB-DLBCL cases displaying 90-100% DNMT1 frequency (Fig. 2D).

3.3 DNMT1 Frequency is Prognostic in R-CHOP-treated DLBCL Cases

OS and PFS according to DNMT1 frequency were tested at every 10% increment and we observed that DNMT1 <20% frequency (n=33; 14.3%) was the cut-off most significantly associated with worse OS (P<0.001, HR: 2.86, 95% CI: 2.38-10.02; Fig. 3A) and PFS (P<0.001, HR: 2.40, 95% CI: 1.78-6.81; Fig. 3B).

Patients negative for DNMT1 expression (n=21; 9.1%) demonstrated significantly worse OS and PFS compared with either strong or weak/moderate intensity immunostaining, and the outcomes did not differ significantly for strong versus weak/moderate intensities (Fig. 3C-D). In

terms of DNMT1 intensity, comparison of patients with strong intensity (n=73; 31.7%) versus the rest of the patients (negative and weak/moderate intensities combined; n=157; 68.3%) was not predictive of OS (*P*=0.127) and PFS (*P*=0.330) (Supplementary Fig. 2A-B). DNMT1-positive cases (n=209/230) showed considerably heterogeneous outcomes with 65-69% (averaged 67%) and 56-60% (averaged 58%) of the patients achieving 5-year OS and PFS, respectively. These indicate that 33% or 42% of DNMT1-positive patients did not achieve 5-year OS or PFS, respectively.

As DNMT1 <20% frequency was prognostic in this series as a whole, we assessed whether it was also predictive of outcomes within DLBCL subtypes. In GCB-DLBCL cases (n=118; Hans subtyping), DNMT1 <20% conferred poorer OS (*P*<0.001, HR: 4.10, 95% CI: 3.43-47.72) and PFS (*P*=0.001, HR: 3.28, 95% CI: 2.22-24.94) (Supplementary Fig. 2C-D) while in non-GCB DLBCL cases (n=112), DNMT1 <20% displayed worse OS (*P*=0.028, HR: 2.03, 95% CI: 1.11-5.64) and not predictive of PFS (*P*=0.088, HR: 1.73, 95% CI: 0.91-4.19) (Supplementary Fig. 2E-F).

Multivariate analysis showed that DNMT1 negativity or DNMT1 <20% frequency was predictive of OS and PFS when analyzed against non-GCB DLBCL subtype or higher IPI scores (≥3), while DNMT1 strong intensity remained insignificant consistent with univariate analysis (Table 2). DLBCL subtyping was significantly predictive of OS and PFS in this series according to both Hans and Visco-Young subtyping algorithms (Supplementary Fig. 3).

3.4 DNMT1 is Frequently Expressed in Mitotic Cells and Significantly Associated with Ki-67 and BCL6 Expression in Primary DLBCL Cases

Although low DNMT1 expression or negativity conferred worse survival, DNMT1-positive cases demonstrated heterogeneous outcomes with a proportion of the cases (33-42%) did not achieve

5-year OS or PFS. In line with this, we observed that DNMT1 was frequently expressed in the mitotic cells where its expression was present in different phases of mitosis including prophase, metaphase and anaphase as illustrated in seven representative cases in Supplementary Fig. 4. DNMT1 intensity was visibly lower than those present in non-dividing cancer cells due to disintegrated nuclear membrane known to occur during early stages of mitosis *i.e.*, prophase,[25] potentially causing diffusion of DNMT1 protein (localized in the nucleus) into cytoplasmic regions.

These observations led us to investigate the potential co-expression of DNMT1 with the proliferation marker Ki-67 involved in cell division and ribosomal RNA synthesis.[26] In reactive tonsil, Ki-67 showed stronger expression in the DZ than LZ in the same lymphoid follicles where DNMT1 was also strongly expressed in DZ compared to LZ (Fig. 4A) and both were expressed in similar population of GC B cells (Fig. 4B). In primary DLBCL cases, both DNMT1 and Ki-67 were expressed in mitotic cells (Fig. 4C).

When compared in DLBCL cases with available Ki-67 staining data (n=155), DNMT1 demonstrated a strong positive correlation with Ki-67 frequency expression (Pearson r =0.60; Spearman ρ =0.61; *P*<0.001; Fig. 4D). Pearson and Spearman correlation matrix comparing the correlation values of DNMT1, Ki-67 and other markers (CD10, BCL6, GCET1, HIP1R, MUM1, FOXP1) in the same set of DLBCL cases (n=155; BCL6, GCET1, HIP1R and MUM1 contained 154 assessable cases) demonstrated that the correlation values between DNMT1 and Ki-67 were the highest (Fig. 4E). Ki-67 frequency expression was not predictive of OS or PFS at every 10% cut-off increment tested in this series of 155 DLBCL cases (data not shown). Interestingly, DNMT1 also showed significantly positive correlation with frequency expression of the GCB-DLBCL marker BCL6 (Pearson r =0.44; Spearman ρ =0.47; *P*<0.001; Fig. 4E).

DNMT1 exhibited significant (*P*<0.05) association with other GCB markers CD10 (r =0.23; ρ =0.21), GCET1 (r =0.22; ρ =0.24), and HIP1R (r =0.28; ρ =0.29) but not significantly associated with non-GCB markers MUM1 (r =0.09; ρ =0.10) and FOXP1 (r =-0.01; ρ =0.02). Finally, within the 155 cases containing Ki-67 scores, only four cases were negative for DNMT1 and their Ki-67 frequencies were 10%, 70%, 80% and 95%. Although three of these cases contained high (≥70%) Ki-67 frequency, we were unable to confirm the association of DNMT1 and Ki-67 in DNMT1-negative cases due to low total number of cases (n=4).

4.0 DISCUSSION

In this study, by using a validated monoclonal anti-DNMT1-2B5 antibody, we showed that DNMT1 was frequently expressed in normal GC B cells and in primary DLBCL cases (n=209; 90.9%). We observed that DLBCL cases negative for DNMT1 expression showed particularly poor survival with less than 30% of these patients achieving 5-year OS or PFS, and they formed the majority of the patients (n=21 of 33) within the DNMT1 <20% frequency subgroup.

Epigenetic heterogeneity, characterized by DNA methylation diversity, is initiated in normal GC B cells, and DLBCL methylome is described as variance from their corresponding cell of origin with higher variability being associated with worse clinical outcomes in DLBCL patients treated with R-CHOP regimen,[8, 27] and increased risk of DLBCL relapse.[28] DNMT1 is the main methyltransferase highly expressed in normal GC B cells and it functions to maintain their methylome stability and prevent DNA damage.[10] Moreover, global hypomethylation is a feature of DLBCL [5-methylcytosine (5-mC) content: 4.9%] compared with normal GC B cells (5-mC content: 12.08%) and patients with higher methylation disruption tend to exhibit ABC-DLBCL subtype.[8] Taken together, low levels of DNMT1 expression or negativity in DLBCL might potentially result in high DNA methylation variability, rendering worse clinical outcomes as supported by our observations of low DNMT1 levels or negativity conferred worse survival in R-

CHOP-treated DLBCL cases. Nonetheless, the prognostic significance of DNMT1 in DLBCL requires validation by independent studies to establish the robustness of its predictive value in R-CHOP-treated DLBCL patients.

DNMT1 was more frequently expressed in GCB-DLBCL cases comparable with the observation that patients with higher frequency DNMT1 expression had better outcomes in this series. Patient cases displaying strong DNMT1 intensity similar to that exhibited by normal DZ centroblasts were more significantly associated with GCB-DLBCL subtype. However, it remains undefined whether GCB-DLBCL cells with strong DNMT1 intensity might originate from centroblast populations in the GC. Among the common subtyping markers, DNMT1 showed the highest positive correlation with the transcription factor BCL6 in our DLBCL cohort. This correlation might reflect roles in a common pathway in GC B-cell biology or a functional relationship between the two molecules. In support of the latter, the pan-DNMT inhibitor 5-azacytidine reduced BCL6 levels and cell viability in the GCB-DLBCL cell line SUDHL6.[29] Moreover, *BCL6* transcription is activated by cytosine methylation of its first intron which inhibits binding of the transcriptional repressor CCCTC-binding factor (CTCF), and DNMT1-mediated hypermethylation of CTCF-binding site of gene loci has been frequently demonstrated.[30-32] Further studies are needed to explore these possibilities.

In terms of the relationship between DNMT1 and Ki-67, higher expression of Ki-67 in the DZ of follicular GCs has been known for over two decades.[33, 34] Higher frequency and intensity expression of DNMT1 in the DZ are consistent with the proliferative expansion known to occur in the DZ of GCs.[35] Recent studies have also demonstrated significant associations of DNMT1 with a higher Ki-67 score in other malignancies including breast cancer (n=348; $P<0.0001$),[36] colorectal cancer (n=14; $P=0.014$) and endometrial cancer (n=48; $P=0.030$),[37] while functional studies of Dnmt1-knockout or knockdown murine models have shown significant decrease of Ki-

67-positive GC B cells[10] and other cell types.[38-40] These results indicate that the frequent co-expression of DNMT1 with Ki-67 in normal B cells appears to be maintained in DLBCL cases, and suggest that DNMT1 enables the proliferation of DLBCL cells in line with our observations of frequent DNMT1 expression in DLBCL mitotic cells as well as the established requirement of DNMT1 in cellular replication.[41, 42] DNMT1 has also been shown to promote cell cycle in leukemia cells,[43] and treatment of RAJI and U-937 lymphoma cells with a selective DNMT1 inhibitor Isofistularin-3 arrested the cells in G0/G1 phases.[44]

An alternative GEP classification termed as consensus cluster classification (CCC) was identified by M. Shipp's group where DLBCL can be classified into three subsets *i.e.* “oxidative phosphorylation” (OxPhos), “B-cell receptor/proliferation” (BCR/proliferation), and “host response” (HR) with similar 5-year survivals (50-60%) in all three subgroups.[45] A total of 2,113 genes (top 5%) were adopted by the CCC algorithm to subgroup the cases and interestingly, these shortlisted genes included *DNMT1* and *MKI67* (encodes Ki-67 protein) where both genes were highly expressed in the BCR/proliferation subset. The BCR/proliferation cluster was characterized by abundant expression of molecules involved in BCR signaling cascade (*e.g. CD19*, *SYK*), cell cycle regulatory genes (*e.g. CDK2*, *CCNB2*) and DNA repair genes (*e.g. H2AX*, *PTIP*). The role of DNMT1 in promoting cell cycle activation has been frequently documented,[46, 47] and Dnmt1 deficiency induces DNA damage of murine GC B cells *in vivo*.[10] These suggest that DLBCL cases co-expressing DNMT1 and Ki67 might represent BCR/proliferation subgroup of DLBCL cases, however, such postulation requires further experimental verifications.

DNMT1 has been proposed as a viable cancer target as it is involved in the proliferation or survival of various malignant cells including breast cancer,[38] renal cell carcinoma,[48] pharyngeal cancer[49] and various other tumors (reviewed by Subramaniam *et al*.).[50] Although very low levels

(<20% DNMT1 frequency) or DNMT1 negativity conferred worse survival, 33-42% of DNMT1-positive cases did not achieve 5-year OS or PFS, DNMT1 was positive in 90.9% of DLBCL cases with majority of them demonstrating ≥60% frequency and its expression was associated with the proliferation marker Ki-67. DNMT1 might thus be considered as a potentially ubiquitous DLBCL target associated with cellular proliferation.

DNMT1 is an FDA-approved druggable protein[51] and discovery of novel specific anti-DNMT1 small molecule inhibitors is an active area of investigation in which high-throughput screening and DNMT1 functional assays have recently produced numerous pre-clinical DNMT1 inhibitors such as Isofistularin-3,[44] antroquinonol D,[52] triclabendazole,[53] laccaic acid A[54] and others.[55-59] In addition, the resolved crystal structures of DNMT1 (Protein Data Bank ID: 3SWR or 4DA4) have enabled characterization of DNMT1 inhibitors' mode of action and precise binding sites.[44, 52, 59] These novel anti-DNMT1 inhibitors might represent future investigation avenues in targeting DNMT1-positive DLBCL cases for destruction, and we suggest that DNMT1 immunohistochemistry with a validated anti-DNMT1 mAb (clone 2B5) can be considered to guide future usage of anti-DNMT1 targeting agents. However, as DNMT1 is also ubiquitously expressed in normal GC B cells, targeting DNMT1 might result in depletion of normal activated B cells and increases the likelihood of immunosuppression as a side effect.

In conclusion, our studies support further investigations on the requirement of DNMT1 for the survival of DLBCL cells as well as the potential of novel specific anti-DNMT1 agents against the proliferation and viability of DLBCL cells.

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Author contributions

S.K.L performed and optimized experiments, analyzed data and wrote the methodologies. E.S.C interpreted staining of DLBCL cases and non-malignant tissues. C.H.L, M.A.A, A.G, M.P.P provided tissue microarrays of the Spanish series of DLBCL cases and their information. M.S.M.S, A.H provided the Malaysian series of DLBCL cases and their information. T.M.G, M.B.B, L.M.P provided tissue microarrays of the Danish series of DLBCL cases and their information. A.H.B provided FOXP1 and HIP1R immunostaining data. K.K.W conceived and designed the research, analyzed data and wrote the manuscript. All authors critically read and approved the final draft of the manuscript.

Disclosure of interest

The authors declare no conflict of interest.

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Table 1: Clinico-demographic characteristics and outcome of DLBCL patients treated with R-CHOP (n=230). ULN: upper limit of normal; HR: hazard ratio; CI: confidence interval. *P*<0.05 is emboldened.

Characteristics	n (%)	OS			PFS		
		HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (years)							
Median (range)	64 (20-91)						
≤ 60	98 (43)	2.73	1.66-4.49	**<0.001**	2.13	1.38-3.30	**0.001**
>60	132 (57)						
Sex							
Female	97 (42)	0.72	0.47-1.10	0.131	0.73	0.49-1.09	0.125
Male	133 (58)						
LDH							
≤ULN	114 (50)	1.63	1.05-2.52	**0.028**	1.83	1.22-2.74	**0.003**
>ULN	116 (50)						
Extranodal involvement							
0-1	193 (84)	1.87	1.12-3.13	**0.017**	1.84	1.14-2.96	**0.013**
≥2	37 (16)						
ECOG status							
0-1	169 (73)	2.59	1.66-4.03	**<0.001**	2.13	1.40-3.23	**<0.001**
≥ 2	61 (27)						
Stage							
I-II	112 (49)	1.54	0.99-2.38	0.054	1.96	1.30-2.96	**0.001**
III-IV	118 (51)						
IPI							
0-2	148 (64)	2.79	1.80-4.30	**<0.001**	2.63	1.76-3.92	**<0.001**
3-5	82 (36)						

Table 2: DNMT1 negativity, frequency and intensity multivariate analysis for OS and PFS in DLBCL patients (n=230) treated with R-CHOP. Non-GCB: Non-GCB DLBCL; Hans: Hans algorithm; VY: Visco-Young algorithm; HR: hazard ratio; CI: confidence interval; *P*<0.05 is emboldened.

	Parameters	OS			PFS		
		HR	95% CI	*P*-value	HR	95% CI	*P*-value
Negative	Non-GCB subtype (Hans)	1.79	1.15-2.78	**0.009**	1.59	1.07-2.38	**0.023**
	DNMT1-negative	2.72	1.47-5.04	**0.002**	2.14	1.16-3.94	**0.014**
	Non-GCB subtype (VY)	2.01	1.30-3.12	**0.002**	1.85	1.24-2.77	**0.003**
	DNMT1-negative	2.62	1.41-4.86	**0.002**	2.05	1.11-3.77	**0.022**
	IPI ≥ 3	2.67	1.72-4.13	**<0.001**	2.53	1.69-3.79	**<0.001**
	DNMT1-negative	2.43	1.30-4.52	**0.005**	1.90	1.03-3.51	**0.041**
Frequency	Non-GCB subtype (Hans)	1.67	1.07-2.60	**0.024**	1.52	1.01-2.27	**0.044**
	DNMT1 <20%	2.67	1.60-4.45	**<0.001**	2.26	1.37-3.73	**0.001**
	Non-GCB subtype (VY)	1.91	1.22-2.96	**0.004**	1.79	1.19-2.68	**0.005**
	DNMT1 <20%	2.61	1.56-4.35	**<0.001**	2.19	1.33-3.62	**0.002**
	IPI ≥ 3	2.81	1.81-4.34	**<0.001**	2.57	1.72-3.84	**<0.001**
	DNMT1 <20%	2.93	1.76-4.88	**<0.001**	2.33	1.41-3.83	**<0.001**
Intensity	Non-GCB subtype (Hans)	1.72	1.09-2.72	**0.020**	1.60	1.05-2.42	**0.028**
	DNMT1 strong	0.81	0.48-1.35	0.409	0.93	0.59-1.46	0.736
	Non-GCB subtype (VY)	1.99	1.26-3.14	**0.003**	1.91	1.26-2.90	**0.003**
	DNMT1 strong	0.85	0.51-1.43	0.538	0.99	0.62-1.56	0.948
	IPI ≥ 3	2.75	1.78-4.25	**<0.001**	2.60	1.74-3.89	**<0.001**
	DNMT1 strong	0.71	0.43-1.17	0.178	0.85	0.55-1.32	0.470

FIGURE LEGENDS

Fig. 1. Immunohistochemical staining of DNMT1 in reactive tonsils and primary DLBCL cases. (A) Reactive tonsil (control tissue) immunostained for DNMT1. The right panel corresponds to the boxed region at higher magnification. (B) Representative DLBCL cases immunostained for DNMT1 with various levels of intensity and frequency. (C-D) DNMT1 frequency (C) or intensity (D; Neg: Negative; W/M: Weak or moderate; S: Strong) distribution in primary DLBCL cases (n=230).

Fig. 2. Relationship of DNMT1 expression with clinico-demographical characteristics and DLBCL subtypes. (A-B) Association of DNMT1 frequency (A; categorical variables) or intensity (B) with clinico-demographic characteristics of primary DLBCL cases (n=230). F: Female; M: Male; LDH: Lactate dehydrogenase; ULN: Upper limit of normal; E/N: Extranodal; ECOG: Eastern Cooperative Oncology Group performance status; IPI: International Prognostic Index; GCB: GCB-DLBCL; NGCB: Non-GCB DLBCL; VY: Visco-Young; Neg: Negative; *: $P<0.05$; **: $P<0.01$; NS: Not significant; (C-D) Comparison of DNMT1 intensity (C; Neg: Negative; W/M: Weak or moderate; S: Strong) or frequency (D; continuous variables) in GCB- or non-GCB DLBCL cases according to Hans or Visco-Young (VY) subtyping algorithms.

Fig. 3. Association of DNMT1 expression with survival in R-CHOP-treated DLBCL cases (n=230). (A-B) Survival according to DNMT1 frequency at 20% cut-off for OS (A) and PFS (B) (HR: Hazard ratio); (C-D) Survival according to DNMT1 intensity for OS (C) and PFS (D) (Neg: Negative; W/M: Weak/moderate; S: Strong).

Fig. 4. Association of DNMT1 expression with Ki-67 or BCL6 protein expression. (A-B) Ki-67 and DNMT1 protein expression in the same region of reactive tonsil; (C) Ki-67 and DNMT1 protein expression in the same case of primary DLBCL with both proteins staining similar

populations of malignant cells and mitotic cells are denoted by arrows; (D) Distribution of Ki-67 frequency according to each 10% increment level of DNMT1 frequency expression in primary DLBCL cases (n=155). Each bar joined by a connecting line denotes the median value of DNMT1 frequency; (E) Correlation of DNMT1 with other markers according to Pearson correlation (top half) or Spearman correlation (bottom half) in primary DLBCL cases (n=155; BCL6, GCET1, HIP1R and MUM1 contained 154 evaluable cases), and significant (P<0.05) correlation values are emboldened and underlined.

Supplementary Table 1: Antibodies and experimental parameters used for IHC. aa: amino acid.

Supplementary Fig. 1. Reactive tonsils immunostained with anti-DNMT1 antibodies: (A) Polyclonal ab19905; (B) Clone 2B5 ; (C) Clone 60B1220.1; (D) Clone 18/DNMT1. The right panel corresponds to the boxed region at higher magnification.

Supplementary Fig. 2. OS (A) and PFS (B) according to strong DNMT1 intensity versus DNMT1 negative, weak and moderate intensity cases (Neg: Negative; W: Weak; M: Moderate; HR: Hazard ratio). Survival according to DNMT1 frequency within DLBCL subtypes stratified according to Hans algorithm in GCB-DLBCL (C-D) or non-GCB DLBCL (E-F) subtype (HR: Hazard ratio).

Supplementary Fig. 3. OS and PFS of GCB- versus non-GCB DLBCL subtypes stratified by Hans (A-B) or Visco-Young (VY; C-D) algorithm. HR: Hazard ratio.

Supplementary Fig. 4. Seven representative DLBCL cases immunostained for DNMT1 at different stages of mitosis (prophase, metaphase, anaphase/telophase).

Fig. 1

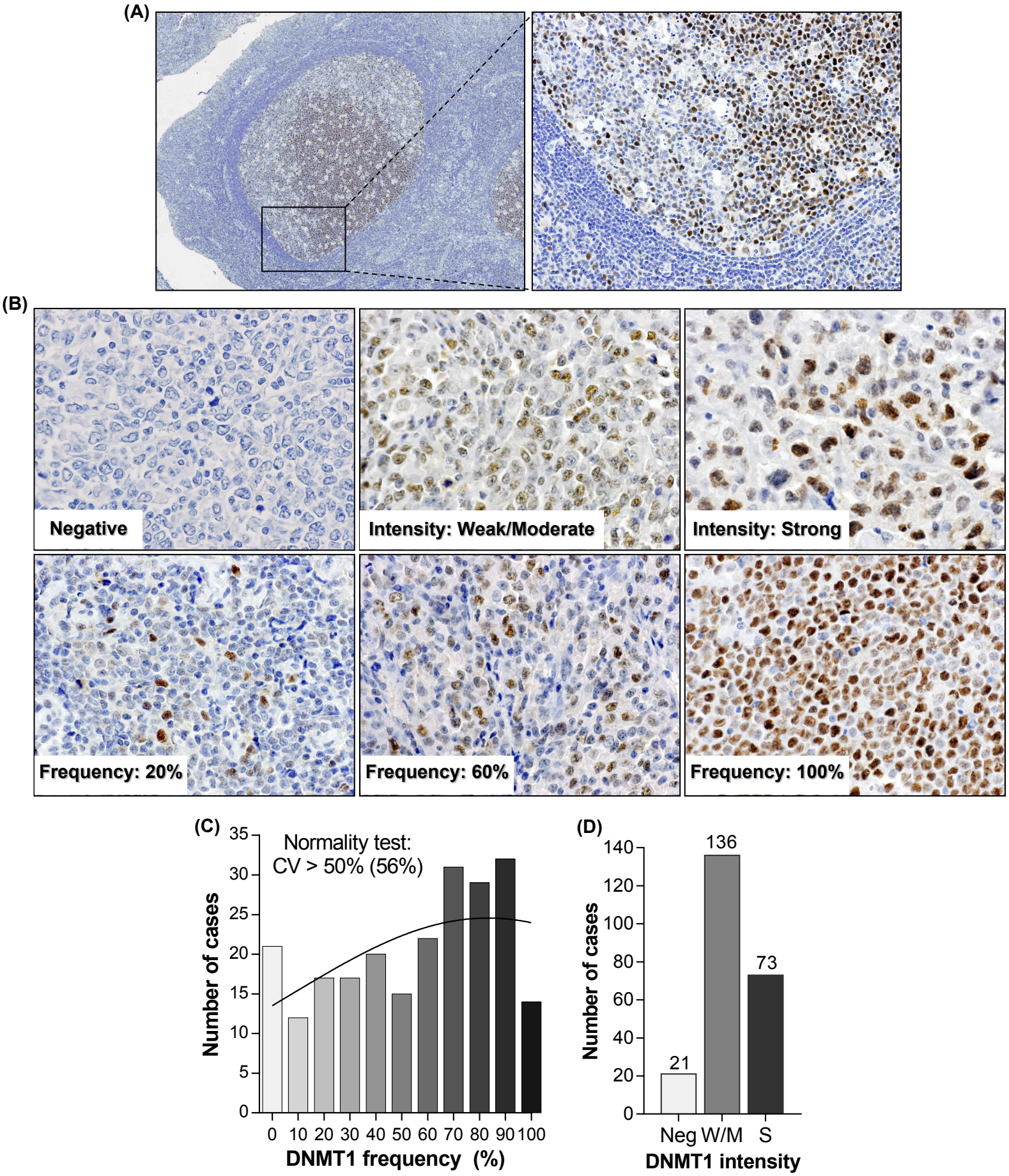

(A)
(B)
Negative
Intensity: Weak/Moderate
Intensity: Strong
Frequency: 20%
Frequency: 60%
Frequency: 100%
(C)
Normality test:
CV > 50% (56%)
Number of cases
DNMT1 frequency (%)
0 10 20 30 40 50 60 70 80 90 100
(D)
Number of cases
136
73
21
Neg W/M S
DNMT1 intensity

Fig. 2

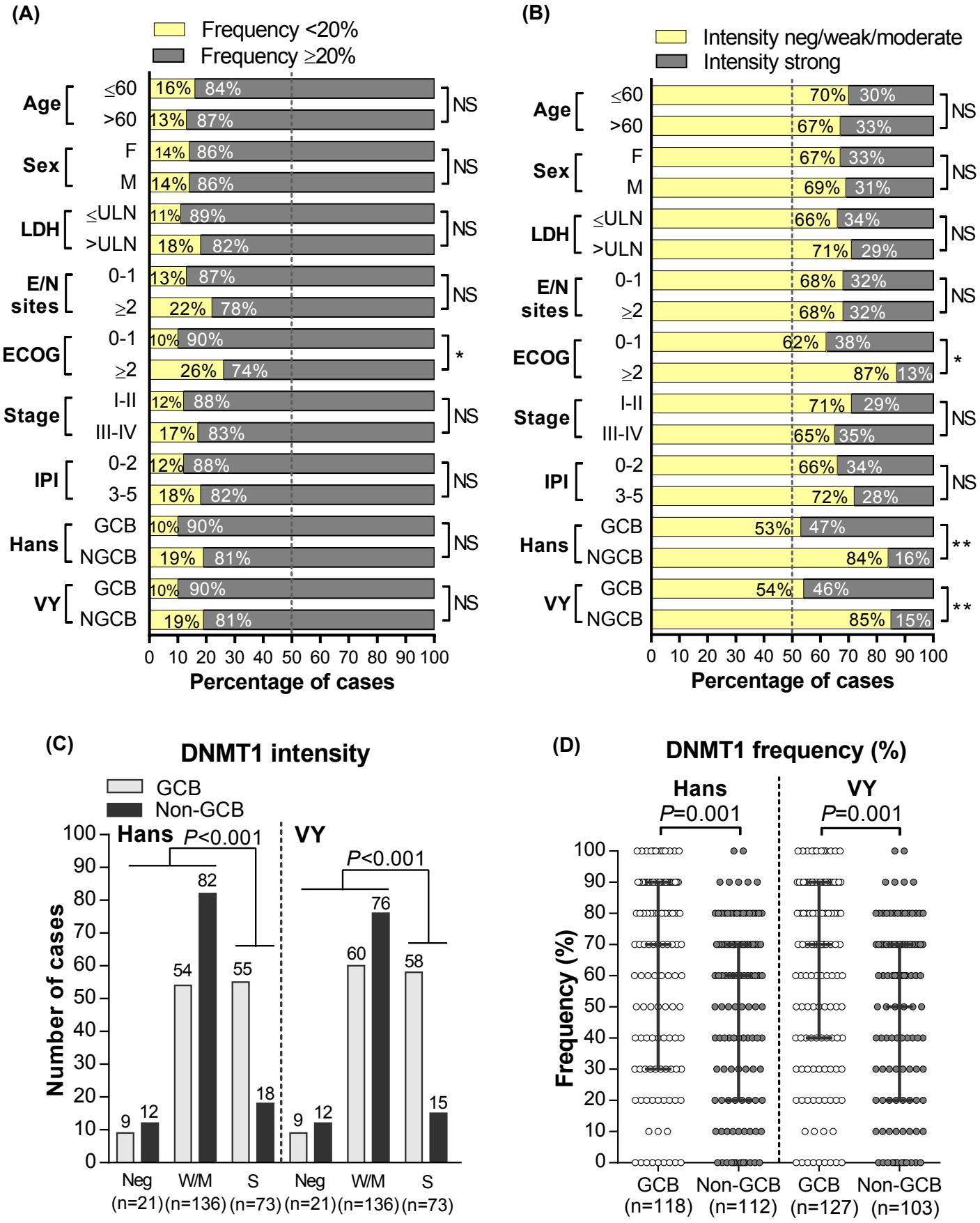

(A)
Frequency <20%
Frequency ≥20%
Age
≤60
>60
16%
84%
13%
87%
NS
Sex
F
M
14%
86%
14%
86%
NS
LDH
≤ULN
>ULN
11%
89%
18%
82%
NS
E/N sites
0-1
≥2
13%
87%
22%
78%
NS
ECOG
0-1
≥2
10%
90%
26%
74%
*
Stage
I-II
III-IV
12%
88%
17%
83%
NS
IPI
0-2
3-5
12%
88%
18%
82%
NS
Hans
GCB
NGCB
10%
90%
19%
81%
NS
VY
GCB
NGCB
10%
90%
19%
81%
NS
0 10 20 30 40 50 60 70 80 90 100
Percentage of cases
(B)
Intensity neg/weak/moderate
Intensity strong
Age
≤60
>60
70%
30%
67%
33%
NS
Sex
F
M
67%
33%
69%
31%
NS
LDH
≤ULN
>ULN
66%
34%
71%
29%
NS
E/N sites
0-1
≥2
68%
32%
68%
32%
NS
ECOG
0-1
≥2
62%
38%
87%
13%
*
Stage
I-II
III-IV
71%
29%
65%
35%
NS
IPI
0-2
3-5
66%
34%
72%
28%
NS
Hans
GCB
NGCB
53%
47%
84%
16%
**
VY
GCB
NGCB
54%
46%
85%
15%
**
0 10 20 30 40 50 60 70 80 90 100
Percentage of cases
(C)
DNMT1 intensity
GCB
Non-GCB
Hans P<0.001
VY
P<0.001
Number of cases
100 90 80 70 60 50 40 30 20 10 0
9
12
54
82
55
18
9
12
60
76
58
15
Neg
W/M
S
Neg
W/M
S
(n=21)
(n=136)
(n=73)
(n=21)
(n=136)
(n=73)
(D)
DNMT1 frequency (%)
Hans
P=0.001
VY
P=0.001
Frequency (%)
100 90 80 70 60 50 40 30 20 10 0
GCB
(n=118)
Non-GCB
(n=112)
GCB
(n=127)
Non-GCB
(n=103)

Fig. 3

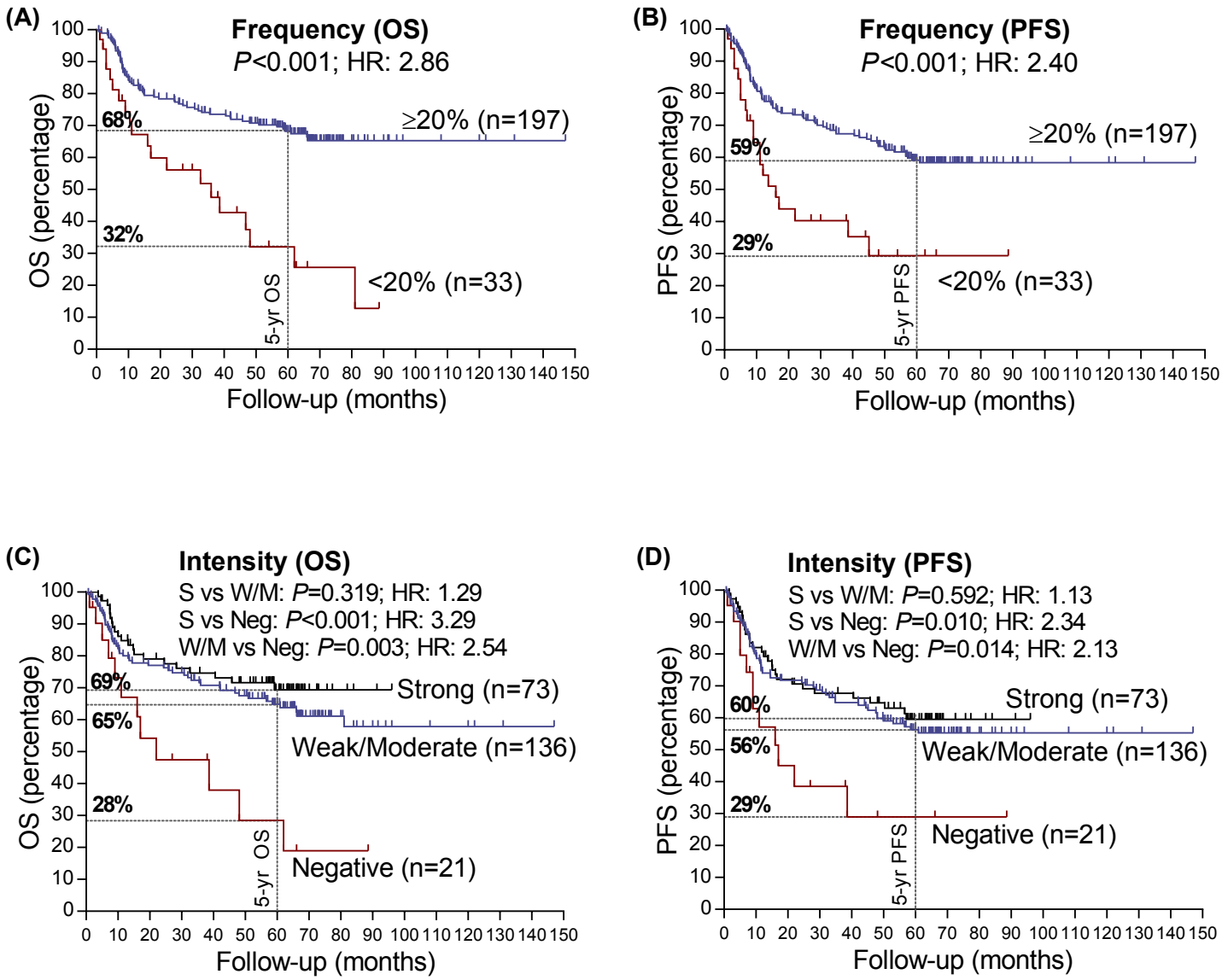
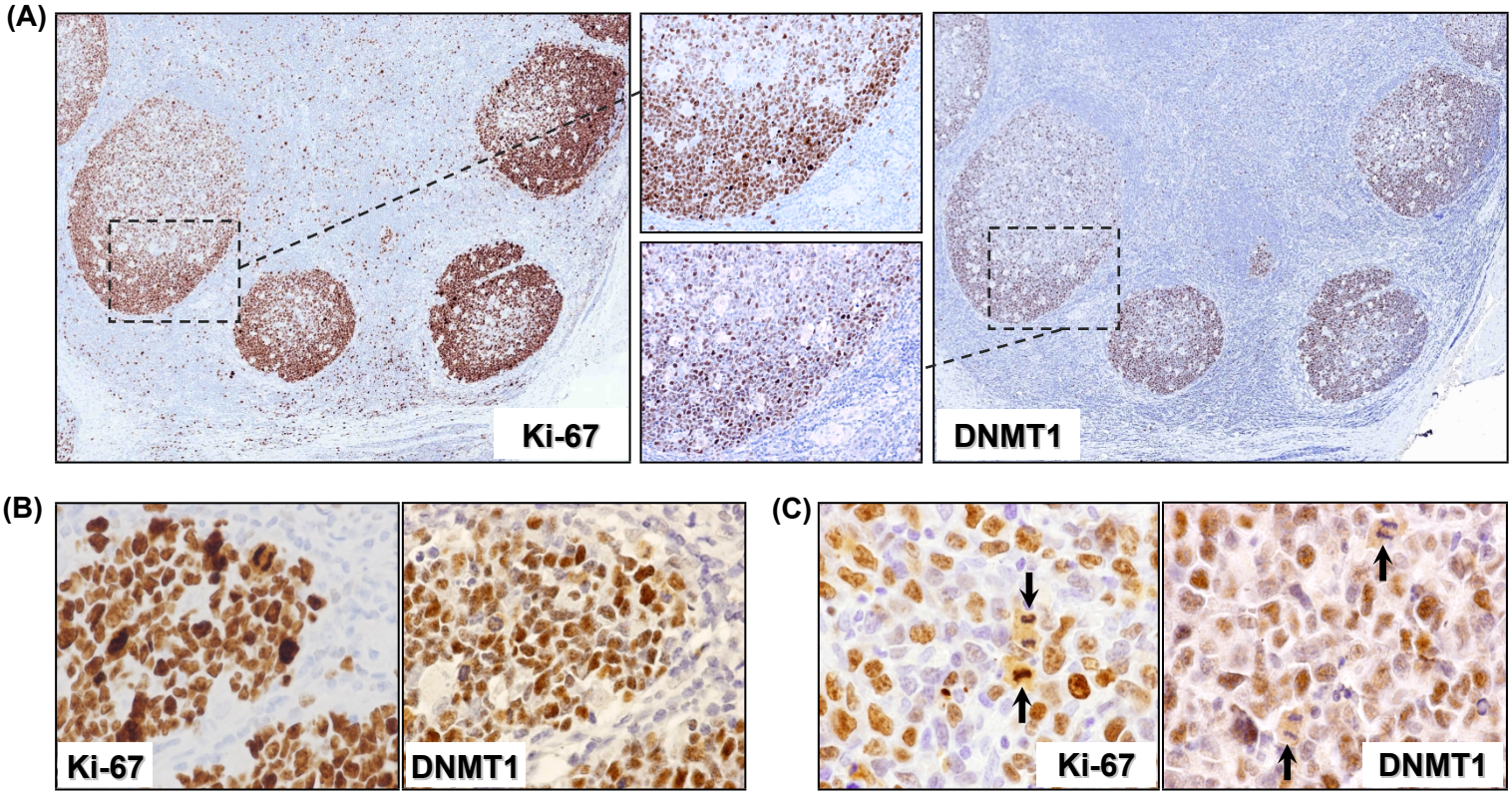
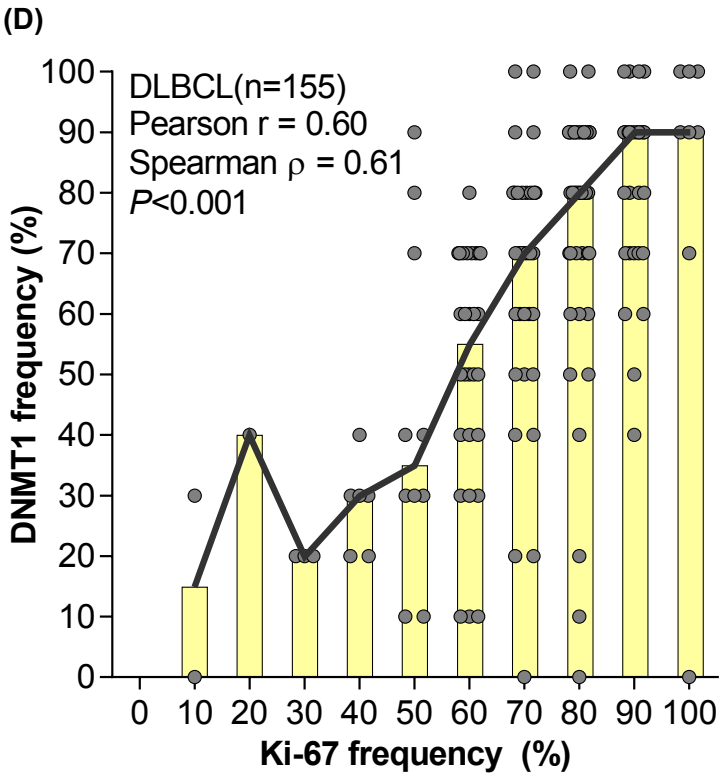
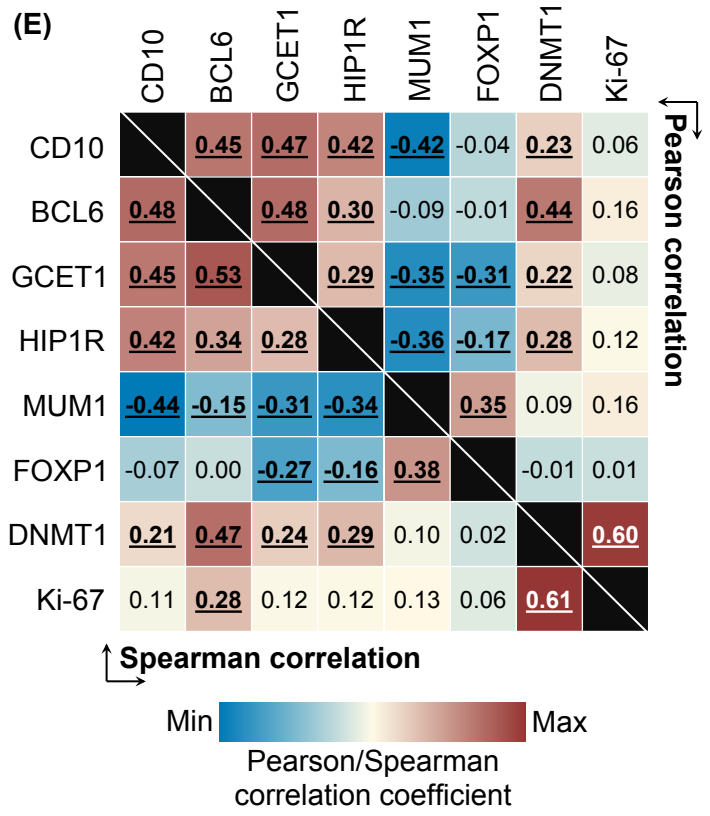

(A)
Frequency (OS)
P<0.001; HR: 2.86
≥20% (n=197)
<20% (n=33)
68%
32%
5-yr OS
OS (percentage)
Follow-up (months)
(B)
Frequency (PFS)
P<0.001; HR: 2.40
≥20% (n=197)
<20% (n=33)
59%
29%
5-yr PFS
PFS (percentage)
Follow-up (months)
(C)
Intensity (OS)
S vs W/M: P=0.319; HR: 1.29
S vs Neg: P<0.001; HR: 3.29
W/M vs Neg: P=0.003; HR: 2.54
Strong (n=73)
Weak/Moderate (n=136)
Negative (n=21)
69%
65%
28%
5-yr OS
OS (percentage)
Follow-up (months)
(D)
Intensity (PFS)
S vs W/M: P=0.592; HR: 1.13
S vs Neg: P=0.010; HR: 2.34
W/M vs Neg: P=0.014; HR: 2.13
Strong (n=73)
Weak/Moderate (n=136)
Negative (n=21)
60%
56%
29%
5-yr PFS
PFS (percentage)
Follow-up (months)

Fig. 4


(A)
Ki-67
DNMT1
(B)
Ki-67
DNMT1
(C)
Ki-67
DNMT1

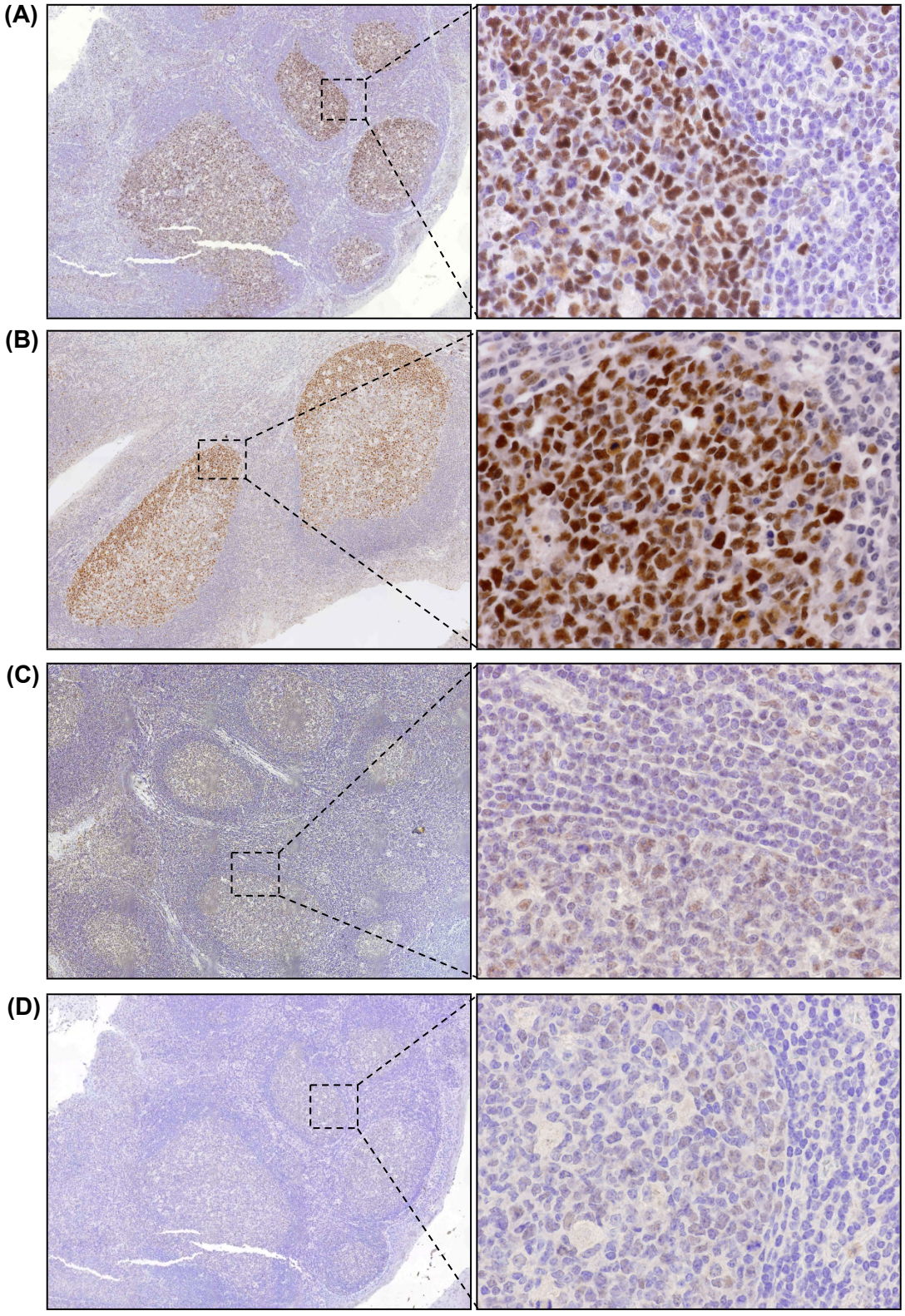

(D)
DLBCL(n=155)
Pearson r = 0.60
Spearman ρ = 0.61
P<0.001
DNMT1 frequency (%)
Ki-67 frequency (%)
0
10
20
30
40
50
60
70
80
90
100


(E)
CD10
BCL6
GCET1
HIP1R
MUM1
FOXP1
DNMT1
Ki-67
Pearson correlation
Spearman correlation
Min
Max
Pearson/Spearman
correlation coefficient

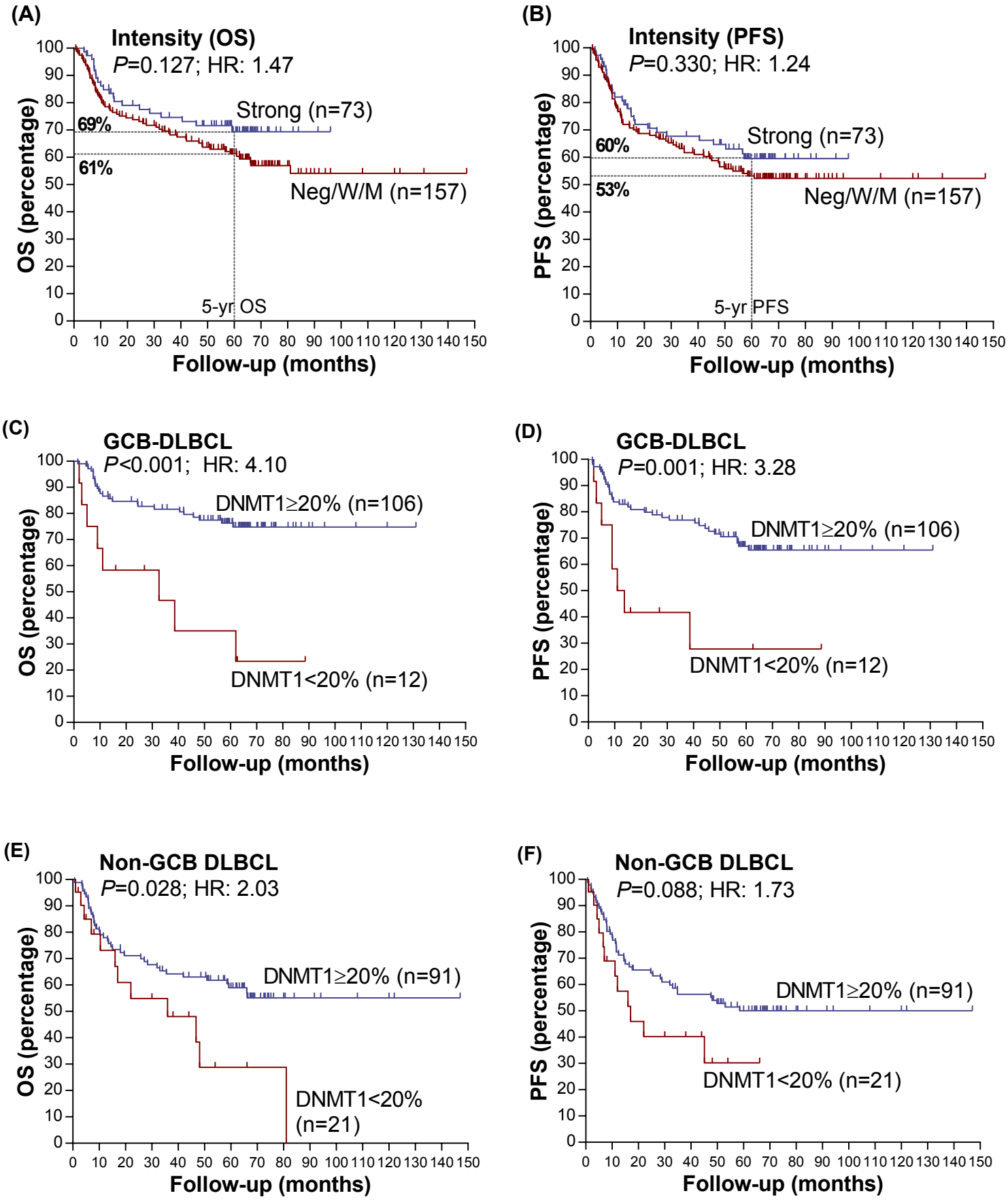
Supplementary Table 1: Antibodies and experimental parameters used for IHC. aa: amino acid.

Protein	Supplier	Host (clone)	Isotype	DNMT1 immunogen	Dilution factor	Antigen retrieval condition	Incubation duration (minutes)
DNMT1	Abcam (Cambridge, UK)	Rabbit (polyclonal)	IgG	100-200 aa	1:500	Tris-EDTA, pH 9.0 (10 min)	30
DNMT1	Abcam (Cambridge, UK)	Mouse (2B5)	IgG1	1-111 aa	1:200	Tris-EDTA, pH 9.0 (10 min)	30
DNMT1	Abcam (Cambridge, UK)	Mouse (60B1220.1)	IgG1	637-650 aa	1:100	Tris-EDTA, pH 9.0 (10 min)	30
DNMT1	BD Biosciences (San Jose, CA)	Mouse (18/DNMT1)	IgG2b	476-670 aa	1:20	Tris-EDTA, pH 9.0 (10 min)	30
Ki-67	Dako (Glostrup, Denmark)	Mouse (MIB-1)	IgG1	-	1:50	Tris-EDTA, pH 9.0 (3 min)	30

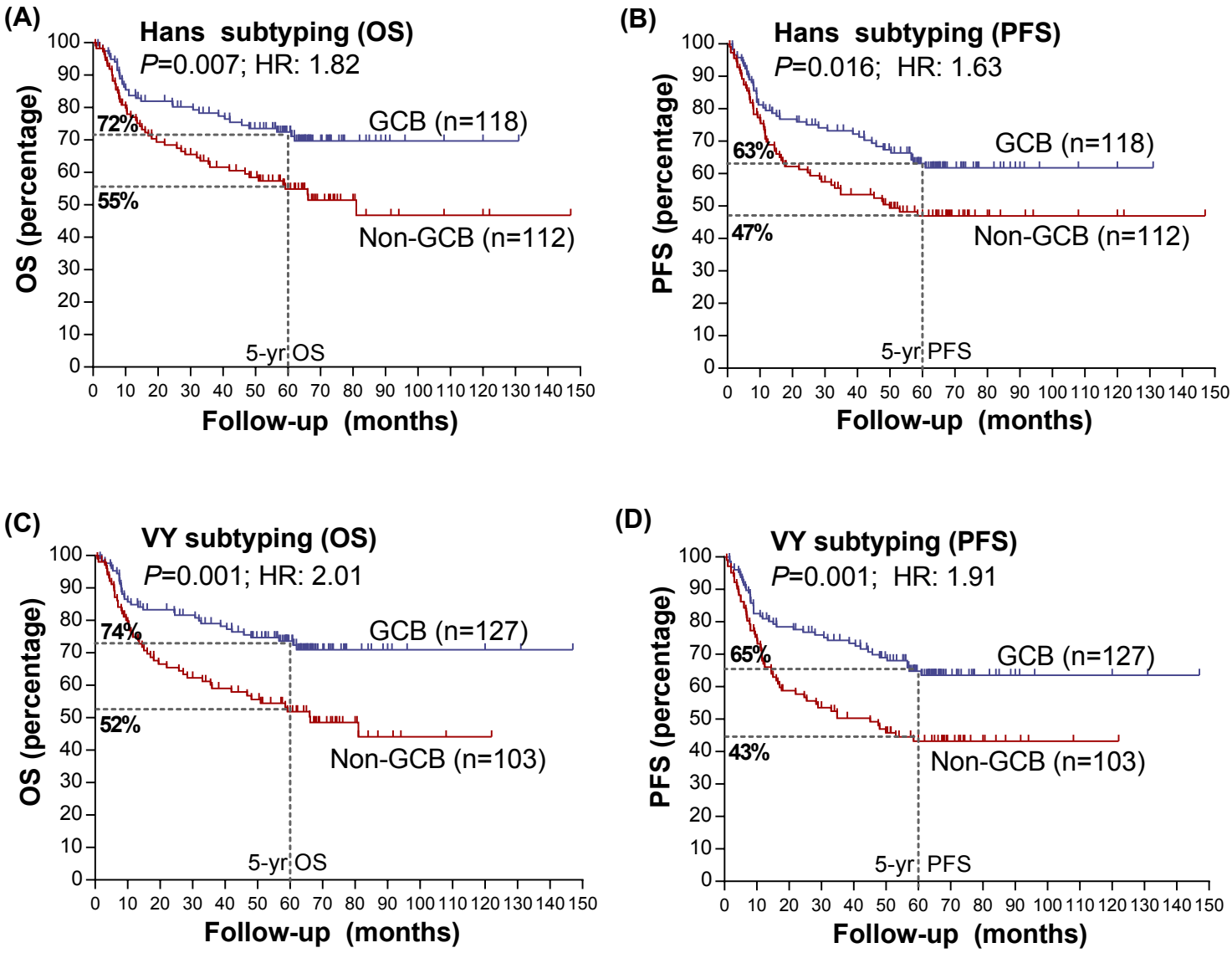
Supplementary Fig. 1



Supplementary Fig. 2



Supplementary Fig. 3



Supplementary Fig. 4